PATENT SPECIFICATION

NO DRAWINGS

1,137,306

1,137,306

Date of Application and filing Complete Specification: 11 July, 1967.

No. 31762/67.

Application made in United States of America (No. 563,953) on 11 July, 1966.

Complete Specification Published: 18 Dec., 1968.

Index at acceptance:—A5 B8

Int. Cl.:—A 61 k 3/62

COMPLETE SPECIFICATION

Inoculum for Poultry

We, RESEARCH CORPORATION, a corporation organized under the laws of the State of New York, United States of America, having a place of business at 405 Lexington Avenue, County, City and State of New York, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to the control of diseases in poultry. In one specific aspect, it relates to a method for the inhibition of the hatching egg transmission of poultry diseases.

Certain diseases such as chronic respiratory disease, avian leucosis and salmonellosis are capable of hatching egg transmission in poultry. The disease producing microorganism, whether it be viral, mycoplasmal, rickettsial or bacterial, passes from the breeding hen via the ovary to the eggs being formed therein, survives the hathcing process and can be detected in the newly-hatched fowl.

Chronic respiratory disease, for example, has a deleterious effect on carcass quality and accounts for extensive condemnation on poultry inspection lines. In spite of the use of antibiotics, vaccines and other measures to control this disease, it is responsible for large economic losses to the poultry industry. The disease passes from the breeding stock to the newly-hatched fowl via hatching eggs making it extremely difficult to break the chain of transmission and control the disease. Even though over 90% of a breeding flock may be immunized using vaccine, the remaining fowl are still able to perpetuate the disease by transovarian passage to their hatching eggs. A similar situation is obtained when antibiotics are employed instead of vaccine for controlling the disease.

When an antigen is introduced into a susceptible host, the host will usually produce an antibody capable of detection by means of a serological test. Any detectable antibody present in a breeding fowl can also be detected in her offspring for several days following hatching.

We have discovered a novel inoculum made up of live microorganisms on or in living animal cells which inhibits transovarian passage of the microorganisms. This inoculum, when introduced into a susceptible or an immune breeding fowl, depressed antibody production and serological titre in the breeding fowl and did not produce a passive antibody in the newly-hatched fowl.

Broadly speaking, the present invention is an inoculum for stabilizing a disease in the parent flock and inhibiting or preventing its transmission via hatching eggs to the flock's offspring. The microorganisms causing the disease is isolated from an infected fowl and grown in an enrichment broth or other suitable nutrient medium. The nutrient medium containing the microorganism is used to inoculate a tissue culture medium containing actively growing animal cells. The resultant infected animal cells are isolated and used to inoculate a fresh batch of tissue culture medium also containing actively growing animal cells. The infected cell culture medium containing living microorganisms is then used to inoculate the flock by administration to individual fowl.

According to the present invention therefore there is provided an inoculum capable of inhibiting the hatching egg transmission of disease in poultry prepared by isolating a disease causing microorganisms consisting of avian leucosis virus or *M. gallisepticum* and growing it in a nutrient medium therefor, inoculating a tissue cell culture medium containing actively growing embryonic avian kidney cells with a portion of the medium containing the microorganism, separating the resultant infected cells, and incubating a fresh batch of tissue cell culture medium containing actively growing embryonic avian kidney cells seeded with the infected cells.

Even though a large portion of the birds in a flock may have already been infected with

[Price 4s. 6d.]

the disease, their blood antibody titre gradually drops to zero on inoculation. After several inoculations the birds in the flock will exhibit a very low or no antibody titre and there will be no clinical evidence of the disease in the inoculated flock. Most important, there is no transfer of the disease to the off-spring from such a stabilized flock.

The invention also provides a method for the inhibition of the hatching egg transmission of avian leucosis and chronic respiratory disease in poultry which comprises isolating a disease causing microorganism consisting of avian leucosis virus or *M. gallisepticum* and growing the microorganism in a nutrient medium therefor, inoculating a tissue cell culture medium containing actively growing embryonic avian kidney cells with a portion of the medium containing the microorganism, separating the resultant infected cells, and incubating a fresh batch of tissue culture cell medium containing actively growing embryonic avian kidney cells seeded with the infected cells to produce an inoculum and administering the inoculum to individual fowl in a poultry breeding flock.

There are three basic steps in the production of an inoculum according to the present invention. The first step is the isolation from the flock of the specific microorganism to be controlled in the flock. The microorganism is preferably but not necessarily isolated from the same flock or from a flock in the same general areas as the flock to be inoculated. The microorganism is grown in an enrichment broth or other suitable nutrient medium and tested for purity using conventional methods. The second step is the addition of the microorganism to a tissue culture medium containing actively growing animal cells in order to prepare infected cells. The third step is the addition of the harvested infected cells to a fresh batch of tissue culture medium containing actively growing animal cells and incubation to produce the inoculum. It is apparent that the nature of the enrichment broth or nutrient medium, tissue culture medium and animals cells employed will depend on the nature of the microorganism used to prepare the inoculum. Our invention is more particularly described with reference to *Mycoplasma gallisepticum*, the pluropneumonia-like organism associated with chronic respiratory disease of poultry. The mycoplasmas are generally classified between the rickettsiae and the viruses because of their morphology, nutritive requirements and close association with animal tissues. Mycloplasmas are capable of growth in cell-free media and the preparation of antigens for their identification is feasible.

The enrichment broth utilized for the isolation of *M. gallisepticum* was prepared as follows: Fifty grams of beef heart was infused for 1 hour with 650 ml of water at 50° C in water bath, heated to 80° C for 2—4 minutes and filtered while still hot. The filtrate was collected in a filtering flask to which had been aded 10 grams of peptone, 10 grams of yeast hydrolysate, 5 grams of dextrose and 5 grams of sodium chloride. The flask was swirled until all the materials had completely dissolved and then cooled to approximately 25° C. The pH was adjusted to 8.3—8.4 with 0.1 N NaOH; approximately 250 ml of NaOH were required. Phenol red indicator solution containing 0.025 gram of the indicator was added and the contents of the flask mixed well and then heated to a slow rolling boil for 2—3 minutes. The medium was filtered hot and 90 ml aliquots were dispensed into flasks and sterilized at 121° C. Penicillin (0.1 gm) and 10 ml of horse serum or bovine serum fraction were added aseptically after sterilization. The medium was free of precipitate and has a final pH of 7.8—7.9.

The medium was dispensed in 2.5 ml amounts into previously sterilized serological tubes contining cotton swabs. When making cultures, the trachea of the bird was swabbed vigorously and the swab placed in the culture tube. Several cultures were made from a large number of birds in the flock to be inoculated. The cultures, with the swabs remaining in the broth tube mixture, were incubated at 37° C for approximately 10 hours. Then 0.7 ml of the contents from each tube was transferred to 4.5 ml of fresh enrichment media in sterile metal capped tubes. After 12 to 24 hours, or on a pH change to 6.8 of the phenol red indicator in the medium, 0.5 ml of the culture was transferred to another tube containing 4.5 ml of fresh enrichment broth. Broth passages were continued as before governed by pH change until the culture was required for the preparation of inoculum. For best results, the number of broth transfers should be kept at a minimum.

At each transfer, plates were inoculated with 0.1 ml of the changing broth culture for colony study. The smallest typical colony of *M. gallisepticum* deeply embedded in the agar was selected for cultivation. The plates were prepared using the same general precedure as for the preparation of the enrichment broth except that the addition of phenol red was omitted. Bacto "certified" agar (other grades leave granular material in the finished product) in a concentration of 0.5—0.7% by weight, was added after the second hot filtration with good mixing. After sterilization at 121° C, the medium was cooled to approximately 50° C. Ten percent sterile horse serum was added aseptically and the contents swirled gently to mix; no penicillin or other bacterial inhibiters were added to the agar medium. The medium was poured into small sterile petri dishes and any air bubbles which formed on the surface of the agar plates were eliminated by passing a flame over the surface.

An avian kidney cell culture was prepared

as follows: avian kidney cells derived from 16—18 day old chick embryo kidney rudiments were placed in an indented trypsinizing flask with 100 ml of a 0.125% by weight solution of trypsin in Hanks balanced salt solution. The kidney cell tissues were trypsinized at room temperature for 10 minutes using a magnetic stirrer and the supernatant liquor was discarded. The kidney fragments were again trypsinized and the cells produced on the second trypsinization were strained through cheese cloth. After centrifugation for 10 minutes at 1000 x g, the supernatant liquid was discarded. After counting, the cells were seeded into a flask containing 100 ml of 199 tissue culture nutrient supplemented by 5% by weight calf serum or 5% by weight horse serium, 0.5% by weight Bacto peptone and 0.12% by weight methylcellulose.

The nutrient can be obtained commercially or prepared according to Merchant. The peptone was added as a stock solution containing 10 gm of peptone dissolved in 100 ml of balanced salt solution. The methylcellulose was added as a stock solution prepared by suspending 4 gm of 15 cps "Methocel" (Registered Trade Mark) (methylcellulose) in approximately 30 ml of balanced salt solution at 90° C and stirring until the powder was thoroughly wetted. The slurry was cooled to 4° C and an additional 100 ml of balanced salt solution, previously chilled to 4° C, was aded with shaking. The nutrient containing added peptone and methylcellulose was dispensed in 50 ml amounts into screw-cap prescription bottles and autoclaved at 120° C for 15 minutes. (The Methocel boiled vigorously during autoclaving and when removed from the sterilizer has the appearance of coagulated protein). The autoclaved nutrient was cooled to room temperature and refrigerated at 4° C overnight. (Methocel becomes more fluid on cooling). Five ml of calf serum was added per 100 ml of nutrient for seeding with avian kidney cells. Five ml of horse serum was added per 100 ml of nutrient for inoculation with *M. gallisepticum*.

The tissue culture nutrient medium was initially seeded with 7.5×10^5 cells/ml and incubated in a gyratory shaker at 37.5° C and 84 cycles/min. At the same time, a sample of 0.5 ml of cells and the nutrient were taken for culture in enrichment broth and agar plates in order to detect any contamination with pluropneumonia-type organisms.

The suspension cell culture was refed 48—72 hours after seeding. At the same time a new kidney cell suspension cell culture, prepared in the same manner as the first, was started. Twenty-four hours after refeeding the cell culture was inoculated with a broth culture of *M. gallisepticum* and returned to the gyratory shaker at 37.5° C for another 48 hours. The suspension culture was then centrifuged at 1000 x g for 8 minutes and the supernatant liquid removed. The infected tissue culture cells were then transferred into the as yet unfected suspension cell culture. (Sufficient nutrient was added to maintain the original cell count of the suspension culture). Since the latter cell culture had been refed 24 hours previously, it continued in an actively growing state. After forty-eight hours further incubation, the cell culture medium was used for inoculation.

The inoculum was administered to the upper respiratory tract in the general vicinity of the trachea at dosages of 0.5 ml per bird to flocks in the filed containing both *M. gallisepticum* serologically positive and negative birds. When the inoculum was administered in repeated doses at intervals, i.e. given at two week intervals, the apparent general health of the flock improved and after the 3rd to 4th inoculation egg production was not depressed. Birds showing a high serological antibody, upon inoculation showed a depression of the antibody. Following the third inoculation in most cases, and beyond the 4th inoculation, no *M. gallisepticum* could be recovered from hatching eggs produced by inoculated breeders. This result was obtained both in the field and in controlled laboratory trials where isolation from non-inoculated positive controls was occurring at the rate of 10—15% as shown in the table below:

Setting Number	Time	Pen Number	Number Eggs Set	Number Embryos Swabbed	Number Positive	Number Negative
1	Before inoculation	7, 8, 9, 10	180	30	3	27
		12	100	10	0	10
2	2 weeks after 1st. inoculation	8, 9, 10	180	23	8	15
	Positive breeder control	7	120	25	6	19
	Negative breeder control	12	132	5	0	5
3	2 weeks after 2nd. inoculation	8, 9, 10	180	29	5	24
	Positive breeder control	7	135	20	0	20
	Negative breeder control	12	135	25	0	25
4	2 weeks after 3rd. inoculation	8, 9, 10	180	20	0	20
	Positive breeder control	7	135	20	2	18
	Negative breeder control	12	135	5	0	5
5	2 weeks after 4th. inoculation	8, 9, 10	180	15	0	15
	Positive breeder control	7	135	25	4	21
	Negative breeder control	12	135	12	0	12
6	2 weeks after 5th inoculation	8, 9, 10	180	25	0	25
	Positive breeder control	7	100	20	3	17
	Negative breeder control	12	100	20	0	20

Serological tests were made on day old chicks of inoculated and uninoculated breeders. In no case were there any positive results from the negative control group (Pen 12). The positive control group showed positives through the sixth hatch except for the fourth hatch which contained a large number of infertile eggs and did not provide an adequate sample for testing. In comparing the control breeders progeny with the inoculated breeders progeny, passively transferred antibody occurs until the third hatch in the inoculated group. Beyond the third hatch the chicks coming from inoculated breeders were serologically negative.

Some 400 breeders were used in the field test. There were 12 hatches from the flock with a total of 1749 hatched chicks. Similar to laboratory trials, no positive serology was obtained beyond the 4th hatch. These chicks were placed on 4 farms and at 11 weeks and 18 weeks the serological test on all chickens were negative.

No inoculum or other treatment was given to the hatched chicks. Cull chicks from second

generation hatching eggs gave negative serological tests. Nor did the continuous piped embryo test commonly used for the detection purposes in the field show any evidence of the disease organism. Similar results were obtained in the third generation. A total of well over 10,000 birds was involved in the field test.

Transovarian passage of avian leucosis virus and hatching egg transmission of avian leucosis is generally known. The method of our invention was tested on two year old breeders negative to pullorum MG and MS tests and susceptible to the NH tumor isolate. The inoculum was prepared using a tissue culture medium infected with NH tumor isolate as described above for *M. gallisepticum*. The inoculum was administered intraperitoneally at a dosage of 0.2 ml of inoculum per bird. Hatching eggs were collected daily and kept for a period of 2 weeks before each setting. Some 300 chicks were produced in four hatches from the inoculated breeders during the course of the test.

No deaths from avian leucosis occurred during the 12 week test period. Necropsy studies at the end of 11 weeks did not indicate any evidence of avian leucosis in any of the inoculated breeders.

Hatching egg transmission of avian leucosis infection was not observed by post mortem examination for gross tumor in progeny during the course of and at the end of the test. Nor did the inoculation of breeders with cellular suspensions infected with the NH tumor isolate produce any observable gross tumors in their offspring during the test.

WHAT WE CLAIM IS:—

1. A method for the inhibition of the hatching egg transmission of avian leucosis and chronic respiratory disease in poultry which comprises isolating a disease causing microorganism consisting of avian leucosis virus or *M. gallisepticum* and growing the microorganism in a neutrient medium therefor, inoculating a tissue cell culture medium containing actively growing embryonic avian kidney cells with a portion of the medium containing the microorganisms, separating the resultant infected cells, and incubating a fresh batch of tissue culture cell medium containing actively growing embryonic avian kidney cells seeded with the infected cells to produce an inoculum and administering the inoculum to individual fowl in a poultry breeding flock.

2. A method according to claim 1 wherein the inoculum is administered in repeated doses at intervals.

3. A method according to claim 1 wherein the microorganism is isolated from the flock to be inoculated.

4. An inoculum capable of inhibiting the hatching egg transmission of disease in poultry prepared by isolating a disease causing microorganism consisitng of avian leucosis virus or *M. gallisepticum* and growing it in a nutrient medium therefor, inoculating a tissue cell culture medium containing actively growing embryonic avian kidney cells with a portion of the medium containing the microorganism, separating the resultant infected cells, and incubating a fresh batch of tissue cell culture medium containing actively growing embryonic avian kidney cells seeded with the infected cells.

5. An inoculum according to claim 4 wherein the disease is chronic respiratory disease, the microorganism is *M. gallisepticum* and the animal cells are 16—18 day old embryonic chick kidney cells.

6. A method for the inhibition of the hatching egg transmission of disease in poultry as claimed in claim 1 substantially as herein described.

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Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1968.
Published by the Patent Office, 25 Southampton Buildings, London, W.C.2, from which copies may be obtained.